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Serotype-specific detection of enterovirus 71 in clinical specimens by DNA microchip array

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Abstract

Enterovirus 71 is an important pathogen that causes high morbidity and mortality in children in Taiwan. Virus isolation in cell cultures has been the standard method for enterovirus 71 identification in Clinical Virology Laboratories. However, virus isolation takes 5–10 days when using cell culture. A microchip for enterovirus 71 detection was developed as an alternative diagnostic method. The novel approach is based on hybridization of amplified DNA specimens with oligonucleotide DNA probes immobilized on a microchip. Two oligonucleotides were used as detection probes, the pan-enterovirus sequence located in the 5′-noncoding region (5′-NCR) and the enterovirus 71-specific sequence located in the VP2 region. The diagnostic procedure takes 6 h. One hundred specimens identified as enteroviruses by viral cultures were tested using this microchip, including 67 enterovirus 71 specimens. The sensitivity of the novel method is 89.6% and its specificity is 90.9%. The enterovirus 71-microchip can detect the amplicon derived from viral RNA corresponding to 1–10 virions in a clinical specimen. Microchip array is a potential diagnostic method for identification of enterovirus in the future.

Keywords: Microchip; Enterovirus 71

1. Introduction

DNA microarray is a highly effective technology of investigating gene expression patterns in diverse organisms (Bowtell, 1999; Duggan et al., 1999) and can be used in such applications as drug discovery, genome mapping, and mutation detection. DNA microarray technology is also highly promising for diagnosing viral infection. The use of DNA microchip arrays for typing and subtyping influenza virus has been reported (Li et al., 2001). A method for species-specific detection of orthopoxviruses in the use of oligonucleotide microchip has also been described (Lapa et al., 2002). It is convinced that the use of DNA chips for detecting pathogens in infectious disease holds great promise. A microchip was designed and developed for diagnosing enterovirus 71.

Among enteroviruses, polioviruses and enterovirus 71 are two major pathogens that can cause severe neuro-

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logical complications. While polioviruses have been eradicated by vaccination, enterovirus 71 outbreaks have occurred frequently in recent years (Alexander et al., 1994; Brown and Pallansch, 1995; Chang et al., 1999a,b; Gilbert et al., 1988; Ho et al., 1999; Komatsu et al., 1999; Shih et al., 2000a; Wang et al., 2002; Wu et al., 1999). The neurovirulence of enterovirus 71 came to attention in 1975 in Bulgaria, where 44 people died of brainstem encephalitis. Epidemics of enterovirus 71, causing CNS diseases, subsequently occurred in New York, Australia, Europe and Asia (Blomberg et al., 1974; Chumakov et al., 1979; Nagy et al., 1982; Schmidt et al., 1974; Shindarov et al., 1979). Taiwan experienced a large enterovirus 71 outbreak in 1998, which infected at least 120 000 children and results in near 80 deaths (Chang et al., 1999b; Ho et al., 1999). Enterovirus 71 infection has been continually occurring in Taiwan recently (Wang et al., 2002). Although the development of antiviral drugs for enterovirus 71 is still in its infancy (Shia et al., 2002), early diagnosis can alert public health workers to take preventive measures, thus limiting the spread of this virus and reducing mortality.

The conventional diagnostic method for enterovirus 71 infection is to isolate the virus from the patient and then identify it using neutralization test or immunofluorescent assay (IFA). However, this method is time-consuming and relies on the availability of anti-sera or commercial monoclonal antibodies. Consequently, molecular diagnostic assays are promising for rapid and sensitive detection of enterovirus 71 (Brown et al., 2000; Singh et al., 2002; Tsao et al., 2002; Yan et al., 2001). This study designed an oligonucleotide microchip for EV71 diagnosis and also evaluated 100 clinical specimens to access the sensitivity and specificity of the diagnostic chip.

2. Materials and methods

2.1. Clinical specimens

One hundred clinical specimens (throat swabs) were tested for enterovirus 71 using the novel microchip. All of the specimens were from Clinical Virology Laboratory, Chang Gung Memorial Hospital, in the years 2000 and 2001. The specimens were collected from patients with clinical features of hand, foot, and mouth disease, herpangina, aseptic meningitis, encephalitis or unspecified respiratory tract infection. All of the specimens were identified as positive for enteroviruses by viral culture and IFA using a pan-enterovirus antibody (Chemicon, Temecula, CA). Furthermore, 67 specimens were identified as positive for enterovirus 71, 23 as coxsackievirus A16, one as echovirus 30, five as echovirus 4, and four as coxsackievirus B4. Type specific antibody (Chemicon) was used for serotype identification. Cell lines used for virus isolation and propagation were RD cells, MRC-5 cells and vero cells. Neutralization assay was conducted with RD cells following the standard procedure for typing enteroviruses (World Health Organization, 1988).

2.2. RNA isolation, cDNA synthesis, and PCR amplification

Viral RNA isolation and purification was carried out using a QIAmp Viral RNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's directions. Briefly, 140 μl of specimen was added to 560 ul lysis buffer, and the mixture was incubated at room temperature for 10 min. 560 μl of ethanol was then added to each sample, and the suspensions were applied to the QIAmp spin column. After centrifuging at 6000 × g for 1 min, the column was washed with 500 μl of buffers AW1 and AW2, and RNA was eluted with 60 μ lelution buffer. Ready-to-Go™ RT-PCR Beads (Amersham Pharmacia Biotech, Inc, US) were used to amplify the viral RNA, and each bead was reconstituted with 19 μl of DEPC-treated water and 1 μl of random hexamer (2.5 ug/μl; Amersham Pharmacia Biotech). Template RNA (30 μl) was immediately added to the appropriate tube for 30 min at 42 °C, and the enzyme was inactivated by increasing the reaction temperature to 90 °C for 5 min. 12.5 μl aliquots of the RT mixture were then used as templates for PCR. The amplification reaction mixtures (25 μl) contained 1.5 U DNA polymerase (Promega, Madison, WI, USA) and EV Mix (11.5 μl; DR. Chip Biotech, HsinChu, Taiwan). Meanwhile, the EV Mix contained two sets of primers (Table 1) and the 5′ end of the reverse primers were biotinylated. DNA template derived from human β-actin (GenBank accession No. m10277, position 2034–2346) and one set of actin primers (15 nucleotides of both ends) were also included in the EV Mix as the PCR internal control. A PTC-100™ Programmable Thermal Controller (MF Research, Inc.) thermal cycler was used to perform the following program: 94 °C for 4 min (1 cycle); 94 °C for 50 s, 55 °C for 50 s, and 72 °C for 50 s (40 cycles); extension at 72 °C for 4 min.

2.3. Hybridization on the EV™ chip

The amplification reaction mixtures contained two sets of primers (both 5′-noncoding region (NCR) and VP2 primers) and the RT-PCR products were hybridized together in the chip. On the other hand, specific oligonucleotides (Table 1) were immobilized at known locations on a polymer substrate to form an enterovirus 71-microchip (DR. EV™ Chip; DR. Chip Biotech). The equal amount of probes Panenterovirus-1, Panenterovirus-2, and Panenterovirus-3 were mixed together and then immobilized at the locations named spots 5. Using

Table 1
Oligonucleotide sequence of primers used for RT-PCR amplification and probes in the microchip

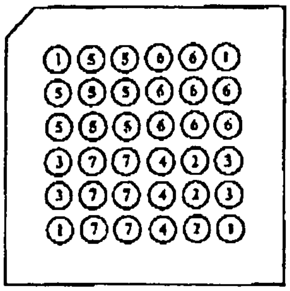
Primer	Sequences (5′→3′)	Location	Genomic position
EVf5	AAGAGYCTATTGAGCTA	5′-NCR	423–439
EVr1	CACYGGATGGCCAATCCAA	5′-NCR	627–645
EVf7	GGITGGTRSTGGAARTTICC	VP2	1179–1198
EVr4	ARRTTIATCCAYTGRTGIGG	VP2	1485–1504
Probe			
Pan EV-1	TCCTCCGGCCCCTGAATGCGGCTAATC	5′-NCR	448–535
Pan EV-2	TGTCGTAACGSGCAASTCYGYRGCGGAACCGAC	5′-NCR	514–546
Pan EV-3	TACTTTGGGTGTCCGTGTTTCITTTTAT	5′-NCR	547–574
EV 71-1	CTTATAAGCAGACTCAACCCGGTGCTGATG	VP2	1390–1419
EV 71-2	TGGCATTCCAATATCACAATTAACAGTG	VP2	1453–1481
EV 71-3	TRCARCACCCGTACGTGCTYGATGCTGGSA	VP2	1429–1458
CA 16-1	CTCGGCACTATCGCAGGAGGGACCGGGAAT	VP2	1343–1373
CA 16-2	CCTACGCCACTACACAGCCTGGTCAGGTTG	VP2	1390–1419
Probe-P	GAGCGGGAAATCGTGCGCGACATCAAGGAG	Actin	2278–2307
Probe-H	ATGAAGCAYGTCAGGGCRTGGATACCTCG	VP1	3201–3229

I, Inosine; Y, C and T; R, A, and G; S, G and C; H, A, C, and T. Genomic Position: relative to the genome of EV71 strain BrCr. Note. The amplification reaction mixtures contained two sets of primers (both 5′-NCR and VP2 primers) and the RT-PCR products were hybridized together in the chips.

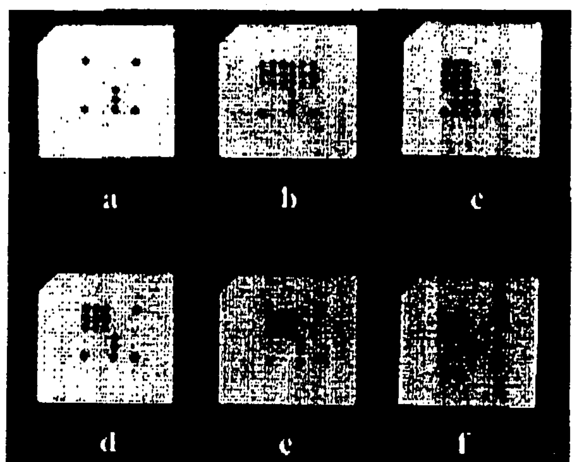
the same method, spots 6 contained the mixture of probes enterovirus 71-1, enterovirus 71-2 and enterovirus 71-3 and the spots 7 contained the mixture of probes coxsackievirus A16-1 and coxsackievirus A16-2. A 8-μl aliquot of the PCR product in a 1.5-ml tube was denatured at 94 °C for 5 min and chilled on ice for 2 3 min. Following denaturation, 392 μl of hybridization buffer (DR. Hyb buffer, DR. Chip; 6 × SSC, 5 × Denhardt's reagent, 0.5% SDS, 100 μg/ml salmon sperm DNA) was added, thoroughly mixed, and placed into the reaction chamber within the DR. EV™ chip. The hybridization buffer also contained 15 nM of biotin-labeled oilgonucleotide that is complementary to hybridization positive control probe (probe-H) listed in Table 1. Hybridization control will display blue color when the hybridization reaction was successful. The chamber was placed in an oven (DR. Hyb™ oven, DR. Chip) for 1 h at 50 °C. The hybridization solution was then discarded, and each chamber was washed five times with 500 μl of washing buffer (DR. Chip, 0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Next, the streptavidin alkaline phosphatase (Promega) was diluted in the blocking buffer (Roach Diagnostics GmbH, Mannheim, Germany) to a concentration of 0.5 μl/ml and added to each chip (400 μl/chip). The chips were then incubated for 30 min at room temperature, and after 30 min of incubation, they were washed five times with 500 μl of washing buffer, and 400 μl of the colorimetric substrate (Roche Diagnostics GmbH, Mannheim, Germany) was added to each chip. The colormetric substrate was prepared by diluting the NBT/BCIP stock solution (Roche) in the detection buffer (0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5) to a concentration of 20 μl per ml. After 10 min of incubation in the dark at room temperature, the chips were washed twice with 500 μl of washing buffer (DR. Chip) and air dried.

3. Results

The enterovirus 71-microchip contains 36 spots arranged in six columns. Fig. 1A shows the contents in the spots. Sequences of probes immobilized at each location are listed in Table 1. Enterovirus 71 specific probes are directed towards the VP2 region of the enterovirus 71 gene. The Pan-enterovirus probes were designed to detect all human enteroviruses. Probe-P, an oligonucleotide with actin sequence, was used for PCR internal control. Probe-H was used for hybridization positive control because that antisense oligonucleotide was included in the hybridization buffer. In order to confirm the serotype-specificity of the enterovirus 71-microchip, probes coxsackievirus A16-1 and coxsackievirus A16-2 that derived from VP2 of coxsackievirus A16 were also immobilized in the chip. The actual hybridization images are displayed in Fig. 1B. Blank sample shows blue color on the spots 1 and 4 that are the hybridization positive control (probe-H) and the PCR internal control (probe-P) (Fig. 1B-a). The amplification reaction mixtures contained two sets of primers (both 5′-NCR and VP2 primers) and the RT-PCR products were hybridized together in the chips. The hybridization pattern of RT-PCR amplicons from enterovirus 71-positive specimen (Fig. 1B-b) exhibits blue color on the spots 1, 4, 5 and 6 (hybridization positive control, PCR internal control, Pan EV probes for all enteroviruses, and



(a)



(b)

Fig. 1. EV71-microchip. (A) The graph represents the microchip elements. The number inside the chip corresponds to control or the probe listed in Table 1. (1), hybridization positive control (probe-H); (2), hybridization negative control (antisense to probe-H); (3), without any oligonucleotide; (4), PCR internal control (DNA fragment of actin, probe P); (5), the mixture of probes PanEV-1, Pan EV-2, and Pan EV-3; (6), the mixture of probes EV71-1, EV71-2, and EV71-3; (7), the mixture of probes CA16-1 and CA16-2. (B) Hybridization patterns of RT-PCR amplicons from blank and five clinical specimens. (a) blank, (b) EV71, (c) CA16, (d) Echo 30, (e) Echo 4, and (f) CB5.

Table 2
The results of 100 clinical specimens by EV71-microchip

Virus	Total number	Positive number
Enterovirus 71	67	60
Coxsackievirus A16	23	1
Echovirus 30	1	0
Echovirus 4	5	2
Coxsackievirus B4	4	0

enterovirus 71-specific probes. Coxsackievirus A16 specimens showed color on spots 1, 4, 5, and 7 (Fig. 1B-c). The other non-enterovirus 71/non- coxsackievirus A16 enterovirus specimens only show color on the control (spots 1, 4) and pan-entero (spots 5) regions (Fig. 1B-d, e, f). One hundred specimens from patients infected with enteroviruses were analyzed (Table 2), all of which had previously been confirmed as enterovirus infection by viral culture. The enterovirus 71-microchip obtained positive results for 60 (89.6%) of the 67 specimens identified as enterovirus 71 by viral culture. Meanwhile, of the 33 non-enterovirus 71 enteroviruses were also tested in this investigation, 3 (9.1%) showed positive for enterovirus 71, including one case of coxsackievirus A16 and two of echovirus 4. Taking the traditional viral culture method as a reference, EV71-specimens that tested positive with the microchip, but negative by the culture method were considered false positive results. Conversely, EV71-specimens that tested positive using the culture method, but negative using the microchip are considered to be false negative results. The numbers of true positive, true negative, false positive and false negative results are 60, 30, 3, and 7, respectively. The novel microchip thus has a sensitivity of 89.6% and specificity of 90.9%.

To determine how many viruses must be present in a specimen before they can be detected by the EV71-microchip, a series of various dilutions of viruses (strain BrCr) were added to a negative specimen. The virus titer was measured by plaque assay. Meanwhile, RT-PCR was performed with RNA samples isolated from each of the serial dilutions. The amount of template RNA corresponding to 10^2–10^3 virions was needed to produce a visible specific amplicon on the agarose gel (data not shown). However, the EV71-microchip can detect the amplicon derived from viral RNA corresponding to 1–10 virions.

4. Discussion

Hand, foot and mouth disease is a common illness among infants and young children. Several enterovirus infections can cause hand, foot and mouth disease, including coxsackievirus A16, A5, A7, A9, A10, B2, B5 and enterovirus 71. However, enterovirus 71 is a major known causative agent which is associated with significant mortality because of its involvement of the central nervous system (Chang et al., 1999b; Gilbert et al., 1988). Rapid diagnosis of enterovirus 71 infection could also assist physicians in distinguishing enterovirus 71 from other enterovirus infections. Making this distinction would enable hand-foot-and-mouth disease or herpangina patients with EV71 infection to receive special care and management, reducing subsequently complications and mortality (Shih et al., 2000b). The enterovirus 71-microchip provides a rapid diagnostic method for enterovirus 71 infection.

Although several PCR-based method for enterovirus 71 identification have been developed (Brown et al., 2000; Shindarov et al., 1979; World Health Organization, 1988), they all require the procedure of viral

culture. Furthermore, since enterovirus 71 is an RNA virus with the existence of quasispecies (Melnick, 1990; Rueckert, 1990), PCR analysis that relied solely on the lengths of the amplified products would be unable to detect minor genomic polymorphism, such as point nucleotide variations, short deletions, and insertions. Gene chip could contain multiple probes and may overcome the limitations of clinical application and provide a potential tool for distinguishing different genotypes of enterovirus 71.

In the present study, three isolates were originally identified as coxsackievirus A16 by immunofluorescent staining with coxsackievirus A16-specific monoclonal antibody, but sequence analysis of the RT-PCR product derived from viral RNA revealed 96% identity with enterovirus 71 and only 65% identity with coxsackievirus A16 in the VP1 region. These three isolates tested positive using the enterovirus 71-microchip.

Similar to other PCR-based methods, microarray chip significantly reduces biological hazard by avoiding the isolation of live virus (Casas et al., 2001; Fox et al., 2002; Manayani et al., 2002; Santos et al., 2002; Verstrepen et al., 2001; Wilson et al., 2000). The relative short length of the amplified fragment in the proposed method also makes it appropriate for immediate hybridization without any fragmentation. The use of biotin labeled primers appears to display a highly efficient incorporation in the final PCR product, and the hybridization procedure takes about 2 h. Including the time required for PCR, the whole procedure takes just 6 h, which is significantly faster the traditional viral culture method.

There are near 70 serotypes of enteroviruses infecting humans. Similarity in antigenic sites causes the difficulty to distinguish certain serotypes of enteroviruses when using antibody in the diagnostic methods. Therefore, the microarray chip based on genomic differences would be a promising tool in enterovirus diagnosis. This study successfully designed and developed an EV71-specific microchip, which may provide a reference to construct a comprehensive microarray chip for other enterovirus identification.

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